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(54) Stable maintenance of nucleic acid in recombinant cells

(57) A generally applicable method is provided for the stable maintenance of heterologous DNA in host cells without requiring the use of defined media or media containing host cell toxins such as antibiotics. In a preferred embodiment, a host cell deficient in the ability to synthesize a cell wall is transformed with a complementary vector that enables the host cell to synthesize a cell wall; conventional hypotonic complex media supply the selection pressure needed to maintain the vector.

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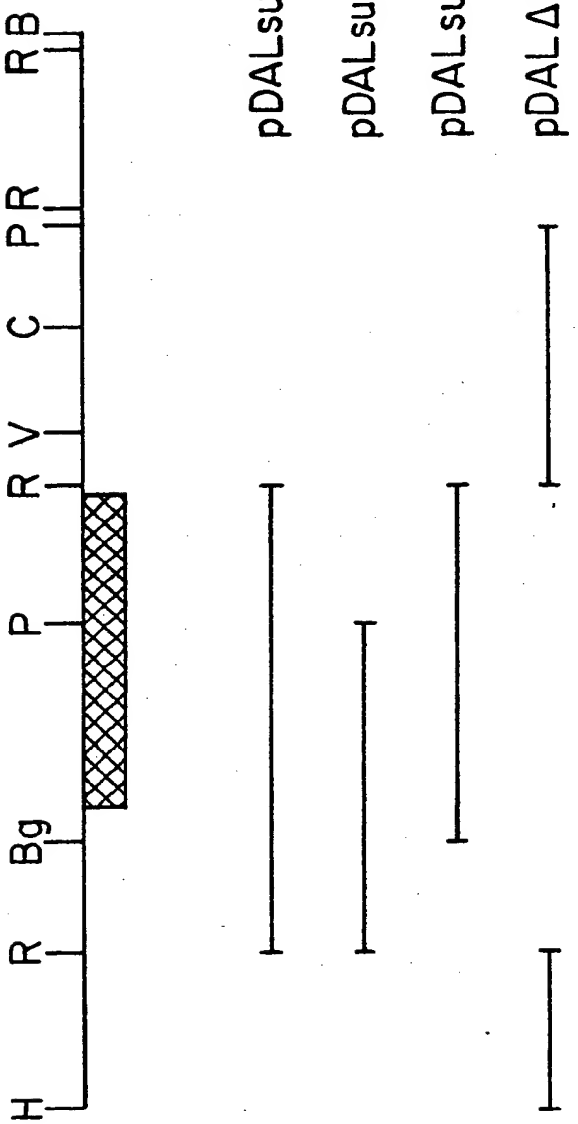
Fig. 1.



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 241 CAGGAAAAAGCC CAGATTGCCAAG GCTTCCTCCTCC GTTACGCTGAAC GGCGAACCGGTA AATCTCGGCTAC ACCATCGGCGCC CTGTCGGATGCA TCATTATCATGG ACATATGACGGC

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 481 TCGTTAGACATC GTTTCCCTTTAG CCTTTAATTTTA GCATGATATGTA AATGATATTGAA TAAAAGCTAGGA AGTGTCGTAATG AGCACAAAACCT TTTTACAGAGAT ACGTGGGCGGAA
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  14 IleAspLeuSer AlaIleLysGlu AsnValSerAsn MetLysLysHis IleGlyGluHis ValHisLeuMet AlaValGluLys AlaAsnAlaTyr GlyHisGlyAsp AlaGluThrAla

 721 AAGGCTGCTCTT GACGCAGGTGCT TCATGCTTGGCC ATGGCCATTTTG GATGAAGCGATT TCACTGCGCAAA AAGGGATTGAAG GCGCCTATATTG GTGCTTGGCGCG GTTCCCCCGGAG
  54 LysAlaAlaLeu AspAlaGlyAla SerCysLeuAla MetAlaIleLeu AspGluAlaIle SerLeuArgLys LysGlyLeuLys AlaProIleLeu ValLeuGlyAla ValProProGlu

 841 TATGTGGCAATC GCTGCTGAGTAT GACGTGACCTTA ACAGGTTATTCT GTTGAATGGCTT CAGGAGGCAGCC CGCCACACGAAA AAAGGTTCTCTT CATTTTCATCTG AAGGTCGATACG
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1201 GGCTTTTTTAAT GCAGTCAGATTC GGCATCGGCATG TATGGCCTTCGC CCGTCTGCTGAC ATGTCGGACGAG ATACCGTTTCAG CTGCGTCCGGCA TTTACCCTGCAT TCGACACTGTCA
 214 GlyPhePheAsn AlaValArgPhe GlyIleGlyMet TyrGlyLeuArg ProSerAlaAsp MetSerAspGlu IleProPheGln LeuArgProAla PheThrLeuHis SerThrLeuSer

1321 CATGTCAAACTG ATCAGAAAAGGC GAGAGCGTCAGC TACGGAGCCGAG TACACAGCGGAA AAAGACACATGG ATCGGGACGGTG CCTGTAGGCTAT GCGGACGGCTGG CTCCGAAAATTG
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1681 AGTATAATGGAA GTAAGAAATCCT TTATTGCAGGTA AATATAAGCAAT TAACTTACCTAA ATGGAGAATTC
 374 SerIleMetGlu ValArgAsnPro LeuLeuGlnVal AsnIleSerAsn OC*
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Fig. 2.

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Fig. 3.

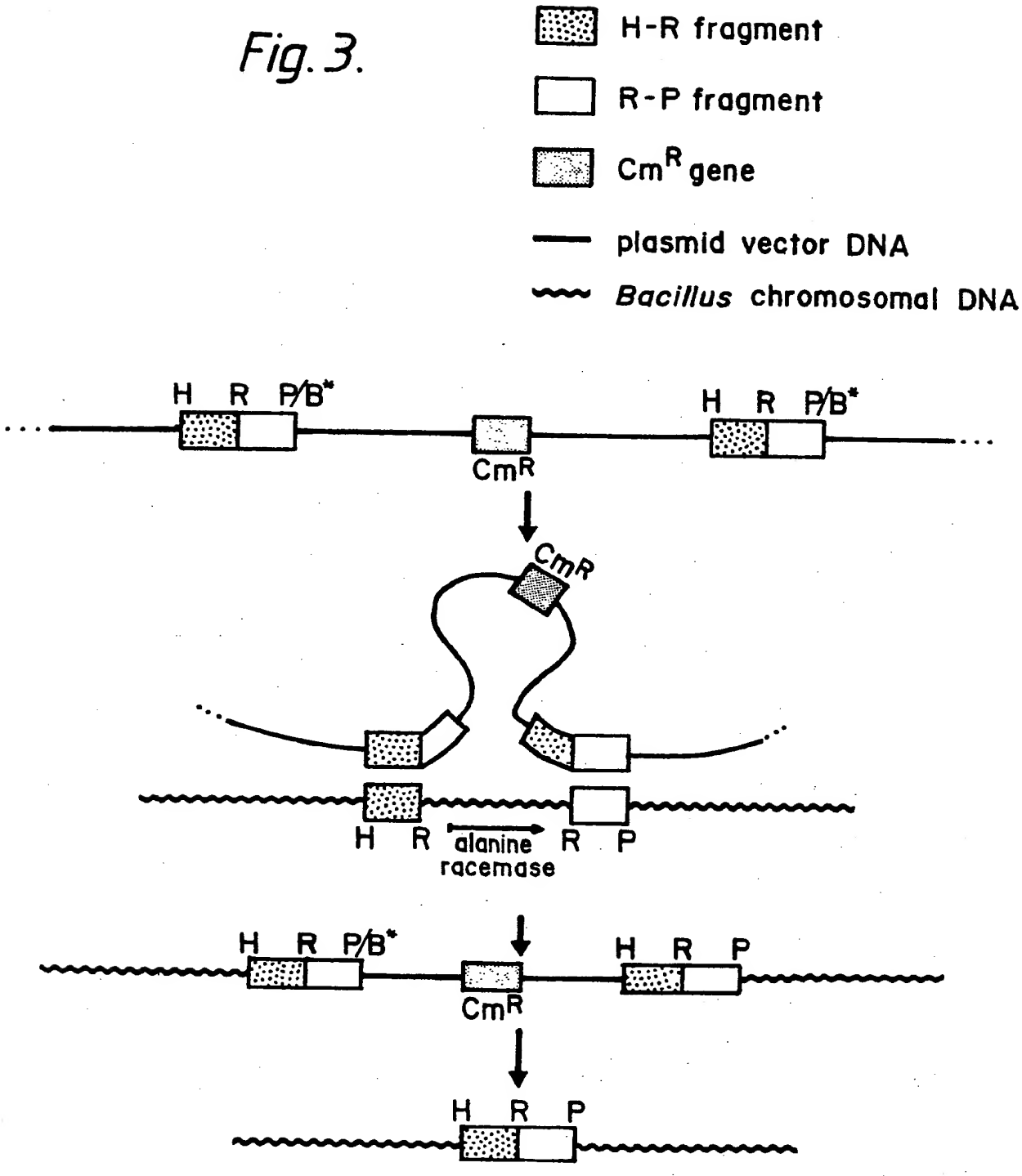

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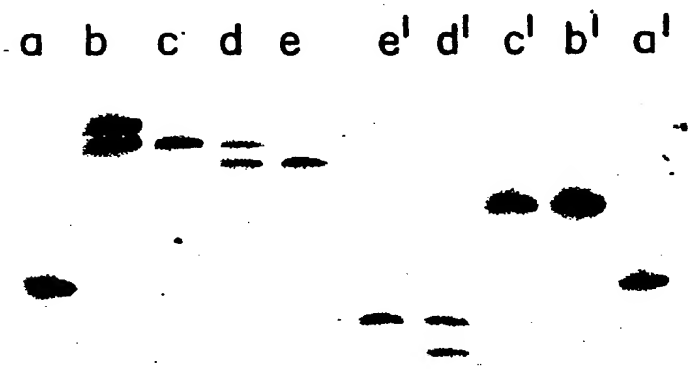
Fig. 4.



SPECIFICATION

Stable maintenance of nucleic acid in recombinant cells

This invention relates to the preparation of desired proteins in recombinant host cells. In particular, it relates to the stable maintenance in host cells of genes encoding such proteins.

Recombinant microorganisms that express an economically desirable protein not needed for growth or survival of the cells hereafter called product proteins, are at a competitive disadvantage in fermentations with other cells that do not express the protein. Such competitive cells include both adventitious contaminants undrelated to the host cells as well as host cells that have lost the capacity to express the desired protein by way of mutagenesis or loss of the transforming vector. The result of competitive overgrowth of non-producing cells is economically serious because the yields of product protein are adversely affected.

Recombinant cells which produce a product protein are transformants with nucleic acid, usually DNA, encoding the protein. This nucleic acid is present on extrachromosomally replicated vectors such as plasmids or is integrated into the host cell chromosome. In either case, the encoding nucleic acid is subject to mutagenic inactivation or loss, e.g. by segregation. Heretofore, this problem has been remedied by associating the nucleic acid encoding the product protein with a selection gene, e.g. by placing both sequences on the same plasmid. If the selection gene (and with it presumably the product protein DNA) is lost the newly bereft former host cell is unable to survive or grow. Selection pressure has traditionally been exerted by specialized fermentation media which included heavy metal ions or antibiotics, substances which are toxic to the cell in the absence of selection genes such as those encoding metallothionein or beta-lactamase, or which media have been depleted or formulated not to contain a substance that is critical to cell growth, but which is synthesized by the transformant through the selection gene. If the selection gene is lost, the cell cannot survive on the medium either because the cell has an auxotrophic requirement or is unable to neutralize a toxin.

Methods for gene maintenance which use specialized media are undesirable. Cell toxins generally need to be removed from the product protein, while it is expensive to prepare defined media or media lacking in a substance needed to satisfy an auxotrophic requirement. The use of antibiotics to selectively maintain plasmids adds to the cost of the fermentation, might require additional purification to eliminate the antibiotic, and even the presence of antibiotics is not always sufficient to maintain the plasmid in some cases[1,2]. Although at least one auxotrophic marker has been used on a plasmid in *B. subtilis*[3], current production techniques for product proteins from bacteria use cheap, complex media which would not be suitable for such selective marker.

A method is known for the stable replication in *E. coli* of plasmids wherein the lambda phage repressor encoded by a plasmid suppresses replication of chromosomally-integrated *E. coli* lambda phage; loss of the plasmid derepresses the phage and the host dies by lysogeny[30]. While this method enables the maintenance of plasmids in hosts grown in complex media without toxins, it is not readily applicable to organisms beyond the host range of lambda phage and presents a risk of reversion by deletion from the chromosome of the lysogenic phage.

What is needed is generally-applicable method and vector for the stable maintenance in transformants of nucleic acid that encodes product proteins, but without the need to formulate specialized media which lack or contain a predetermined substance.

Accordingly, it is desirable to povide a generally applicable method for maintaining recombinant nucleic acid in transformant cells by the use of cells capable of growth on inexpensive complex or undefined media but which are incapable of growth or survival in such media upon loss of the transformed phenotype.

It is further desirable to provide host-cells that are incapable of reverting to the parental phenotype.

According to the present invention there is provided a method comprising providing a host cell which is unable to grow or survive in a hypotonic complex medium free of substances which are toxic to the host cell; providing a vector encoding (a) a complementary protein that enables the cell to grow and survive in such medium and (b) a product protein: transforming the host cell with the vector; culturing the host cell on the medium; and recovering the desired protein from the culture.

One specific embodiment is a method comprising providing a host cell which is unable to synthesize a cell wall constituent; providing a vector encoding (a) a protein complementing the genotypic definiency of the host cell, thereby enabling the host cell to synthesize the cell wall constituent, and (b) a desired protein; transforming the host cell with the vector; culturing the host cell; and recovering the desired protein from the culture.

Vectors are more reliably maintained within the host cells if genotypic deficiency is introduced by a method comprising providing a bacterial cell which enzymatically synthesizes a cell wall, and mutating the genome of the cell in order to create a predetermined deletion in the cell DNA responsible for the expression of an enzyme necessary for the synthesis of the cell wall, whereby the cell is rendered phenotypically unable to synthesize peptidoglycan. Host cells produced by this process will not revert, unlike the point mutations characterizing such mutants in nature. Specifically provided is a host cell having a deleted D-alanine racemase gene.

The primary advantage of this system is that it provides selection of plasmid maintenance in complex media without the addition of antibiotics. Although other auxotrophic markers have been placed on plasmids to allow selection, these have been for amino acid biosynthetic enzymes, which necessitate growth in minimal media[3]. An additional advantage of the use of enzymes involved in cell wall metabolism is that the loss of

enzyme activity leads to cell lysis[4-6], preventing the accumulation of a population of cells which have lost the plasmid. This is essentially a passive strategy for plasmid maintenance, killing cells which have lost the plasmid, rather than improving the segregation of the plasmid. An alternative strategy for plasmid maintenance in *B. subtilis* has been described which involves the use of DNA fragment which apparently acts to promote the proper partitioning of plasmids in *B. subtilis*. This method has been used to stabilize a segregationally unstable plasmid in *B. subtilis*[2]. However, it is not certain that such a method would be effective in preventing segregation under all conditions, and if segregation does occur, there is no selection against cells that have lost plasmid, save antibiotic resistance. The passive strategy outlined herein could thus have an advantage over the use of a partitioning factor.

Brief description of the drawings

Figure 1 depicts a restriction map of the pDAL1 insert obtained in Example 1. The position of the structural gene for D-alanine racemase is indicated by the hatched box. The direction of transcription of the gene is from left to right. The subcloned fragments used to construct the plasmids pDALsub1, pDALsub9, pDALLsub6 and pDALdeltaR1 are indicated on the figure. The restriction sites are abbreviated as H, HindIII; R, EcoRI; Bg, Bg1II; P, PvuII; V, EcoRV; C, ClaI; B, BamHI.

Figure 2 is the DNA and amino acid sequence of a D-alanine racemase gene from *B. subtilis*. The sequence for the 1700 bp EcoRI fragment of pDALsub1 is shown, together with the predicted amino acid sequence for a protein starting with the ATG at nucleotides 562-564.

Figure 3 describes the strategy for deletion of the *dal* structural gene from *Bacillus*. The population of plasmid pDALdeltaRI contains concatemer species shown in the top line. This concatemer recombines with the *B. subtilis* chromosome due to the homology of fragments H-R and R-P, as shown in the second line. This results in the loss of the *dal* structural gene and the direct repeats of the H-R and R-P fragments flanking the integrated plasmid and Cm^r gene, shown in the third line. The resulting phenotype is Dal-, Cm^r. Recombination between the repeated BE fragments in the absence of selective pressure for Cm^r (growth on Cm-free media) leads to loss of the integrated plasmid and a Dal-, Cm^s phenotype, shown in the bottom line.

Figure 4 is a DNA blot hybridization analysis of the delta *dal*-2 mutation described *infra*. The 2.7 kb HindIII-ClaI fragment of pDAL1 (see Figure 1) was isolated, labelled by nick translation, and used as the hybridization probe. Lanes: a, a', 1.7 kb EcoRI fragment of pDAL1, ; b,b', BG2189::pDAL1 isolate used to rescue pDAL1 in *E. coli;* c, c', GB2036; d,d' BG2189 (trpC2, *dal*::pDALdeltaRI); e,e', BG2190 (trpC2, delta *dal*-2). Lanes b-e digested with HindIII, lanes b'-e' digested with ClaI.

Detailed description

The host cells used herein are unable to grow or survive in a hypotonic complex medium that is free of substances which are toxic to the untransformed host cell.

A hypotonic medium is one having an osmolality which is sufficiently low to lyse protoplasts of untransformed host cells. Generally, this osmolality will be lower than that of the host cell protoplasm. However, microbial protoplasm varies considerably in its osmolality. For example the internal tonicity of *E. coli* has been reported to be 0.6 osmolal while that of a *Micrococcus* species 1.0 osmolal[31]. Variations in growth conditions and media components also affect the ability of host cells to survive in hypotonic media. Accordingly, designation of a medium as hypotonic will be host cell specific, but is readily determined by the lysis of untransformed host cell protoplasts upon suspension in the candidate medium.

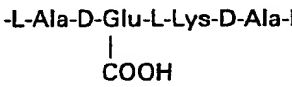
Complex media are defined herein as compositions for supplying carbon, nitrogen, trace ions and other growth factors required by the intended host which have been prepared by a process that entails neither adding nor depleting any predetermined substance required for growth or survival of the untransformed source in the absence of the vector bearing the selection gene nor adding an exogenous cell toxin such as an antibiotic or metal ion. Obviously, complex media (as defined herein) will contain predetermined growth factors such as amino acids or vitamins where required for growth of the host cell provided, however, that the requirement is not the one to be supplied by the expression of the selection gene from the vector. While it is not preferred that a host cell have any nutritional requirement beyond that which is to be ameliorated by the selection gene, this does not present as severe an economic problem as the need to remove from complex media a substance satisfying an auxotrophic requirement.

Preferably, the nitrogen source used for the host cells is undefined and of nonbacterial origin. The term "an undefined nitrogen source" uses the adjective "undefined" to mean that the nitrogen required for cell growth is not supplied by a source of combined nitrogen that is lacking in a particular nitrogen-containing compound. Thus, for example, soybean meal or yeast hydrolysate falls within the definition, whereas a medium does not if it contains as its nitrogen source a hydrolysate that is depleted in a predetermined amino acid or which consists of a mixture of crystalline amino acids. In the preferred embodiment herein the D- isomer of alanine is not found in common complex media from non-bacterial sources, making the conversion of L- to D- alanine by D-alanine racemase essential for cell growth. Lack of the ability to synthesize D-alanine leads to rapid cell lysis due to an inability to form cell walls[4-6].

Suitable host cells generally are bacteria, including both gram negative and gram positive genera, although in principle the method herein is applicable to eukaryotic cells such as yeast and fungi. All that is needed is a host cell that is unable to survive in unspecialized complex media as described above and which is permissive

to recombination with a vector bearing DNA encoding a protein that enables growth and survival in such media.

Among the bacteria, suitable host cells include those in which one or more enzymes are deficient or inactive, these enzymes being necessary for the assembly of the peptidoglycan cell wall or for synthesis of precursors used therein. The synthesis of peptidoglycan has been extensively studied. Four stages in biosynthesis can be distinguished: (a) formation of a disaccharide intermediate, GlcNAc-MurNAc-(pentapeptide)-PP-lipid; (b) modification of the



peptide moiety by amidation of the alpha-COOH of D-glutamic acid and introduction of a pentaglycine bridge unit; (c) polymerization of amidated disaccharide-pentapeptide-pentaglycine units to form linear, non-crosslinked peptidoglycan strands; and (d) closure of pentaglycine bridges by transpeptidation to form the final cross-linked polymer. In addition, D-alanine, D-glutamic acid and the D-alanyl-D-alanine dipeptide are all critical peptidoglycan precursors. Any host cell is suitable that is deficient in enzymes required for the synthesis of the precursors or for the assembly of the peptidoglycan structure. Examples of such enzymes include D-glutamic acid and D-alanine racemases. D-alanyl-D-alanine ligase and N-acetyl-L-diaminopimelate deacylase. The ligase could be particularly advantageous in that it would eliminate the possibility with the racemases that untransformed cells could grow in mixed culture with transformants by cross-feeding. Also, one can use D-amino acid containing media with such host cells. Other enzymes will be apparent to the ordinary artisan or will be identified in the future. Bacterial strains which are deficient in D-alanyl-D-alanine ligase and N-acetyl-L-diaminopimelate deacylase[6], as well as D-alanine racemase[29, 12,13,14] are known. Suitable strains also are obtained in accordance with methods known *per se* using chemical or ultraviolet mutagenesis of protoplasts in isotonic culture media followed by assay on replica plates for strains unable to survive in hypotonic culture media. Methods and media are well known in the art for producing and propagating protoplasts (bacteria not containing cell walls). See for example EP 138,075A.

Once host has been identified that is unable to survive under unspecialized conditions, e.g. that is not capable of forming a cell wall, it is necessary to secure DNA encoding a protein that removes the host disability. This invention is enabled without even knowing thename or the nature of the protein encoded by the DNA. Separate aliquots of the genome of the parent organism from which the mutant was obtained are digested with a bank of restriction enzymes so as to produce a plurality of genomic fragments ranging about from 500 to 3000 kb. These fragments are separately ligated into plasmids to produce a genomic library in accord with methods which are conventional in the art. The library plasmids are cloned, isolated and then used to transform the deficient mutant. Transformants that survive the removal of specialized culture conditions by complementation with library DNA represent suitable host-vector systems for use herein. This is a routine screening procedure. In addition, DNA which encodes D-alanine racemase is known and has been cloned[12,13,14]. Plasmids bearing and expressing such DNA are able to complement D-alanine racemase-deficient mutants and enable their survival in complex media.

The selection gene is defined in terms of the host cell deficiency. If the host is dificient in an enzyme, the selection gene will encode an enzyme having the activity of that which is deficient in the host. Ordinarily, but not necessarily, the selection gene is the normal host cell protein that was mutationally inactivated in generating the dificient host. In the counterpart to the preferred host, the selection gene is D-alanine racemase.

Suitable product proteins are diverse, and include proteins of prokaryotic as well as eukaryotic origin. The preferred proteins are industrial enzymes such as fungal or bacterial proteases, including subtilisin, and mammalian proteins such as interferon or bovine rennin. The absense of any need to remove toxins such as antibiotics or metal ions from productive cultures is particularly advantageous with large volume industrial protein which are intended for use in enzymatic processing or as food or laundry additive where the proteins are not ordinarily purified to high degree and, in many cases, where whole cells are employed. The desired proteins ordinarily are heterologous to the host cell, heterologous meaning that the protein is not expressed by the host cell under the conditions of culture of the transformant. Most heterologous proteins are of mammalian origin. However, great improvements in the yield of homologous proteins are made possible by the use of recombinant technology, e.g. by increasing the gene dosage by placing the desired gene on a high-copy number plasmid or by the use of stronger promoters than those that normally control the expression of the protein in the host, so that vectors bearing DNA encoding desired homologous proteins are included within the scope hereof.

The DNA encoding the selection gene and protein product is introduced into the host cell by way of a vector. The vectors to be employed herein are those commonly available in molecular biology, generally plasmids, for which the intended host is permissive. The preferred expression vectors are prokaryotic plasmids containing the selection gene under the control of a promoter recognized by the intended host. Recognition means that the selection gene is transcribed by the host. There is a certain amount of interspecies recognition of promoters among bacteria. For example, *B. subtilis* promoters are generally recognized by *E. coli*. As applied here, this means that pDalsub1 will complement D-alanine racemase deficiency in *E. coli*. In any case, it would

be routine to insert a host recognized promoter 5' to the ATG of the structural component of the selection gene. Furthermore, the vectors generally will contain an origin of replication recognized by the intended host, although this will not be necessary where a nonreplicative expression vector is incorporated into the host chromosome byhomologous recombination. In this case the vector will contain DNA that is homologous (capable of hybridizing) to a portion of the host genome. Expression of chromosomally integrated plasmids is well known in *Bacillus*. While the vectors generally will be plasmids, phage also are used.

The vector also comprises nucleic acid encoding the product protein under the control of a host-recognized promoter. This promoter is advantageously a strong promoter such as the tac, spac-I promoters or alkaline phosphatase promoters. The product protein promoter may be the same as, or different from that which controls the expression of the structural selection gene.

The method of this invention provides a host that is not capable of growth or survival on complex media and a vector that, upon transformation of the host, expresses a protein enabling growth on complex media without the addition of a toxin such as an antibiotic. Growth or survival means that the objectives of this invention are obtained if host cells that have lost the vector simply become incapable of further replication and therefore do not grow, whereupon the actively growing transformants will overgrow any cells in the static state. However, it is preferable that the untransformed revertants die rather than become static since this will increase the cell density of producing cells. That is why the preferred embodiment herein is the use of a peptidoglycan synthesis-deficient host. When bacteria are unable to synthesize peptidoglycan they swell and rupture in the hypotonic complex media conventionally used in fermentations. This is the same mechanism by which many antibiotics exert selection pressure in recombinant fermentations. However, the method of this invention makes it possible to eliminate the antibiotics from the culture medium and achieve essentially the same physical results, i.e. cell lysis.

In reference to the following Examples, *E. coli* MM294 was cultured in LB medium and transformation was carried out by the procedure of Dagert and Elrich with selection on LB plates containing either 12.5 microgram/ml of Chloramphenicol (Cm) or 50 microgram/ml ampicillin. *B. subtilis* was cultured in the following complex media from Difco: Penassay antibiotic medium 3 (PAB), Triptose Blood Agar Base (TBAB), Nutrient Broth (NB), and were used as indicated in the text. Cm^r colonies were selected on TBAB plates containing 5 microgram/ml of drug. Bacterial transformation procedures and the minimal medium used in certain experiments have been described elsewhere[17], as well as PBS1 lysate preparation and transduction[18]. Two cloning vectors have been used: pBS42, a *B. subtilis-E. coli* shuttle vector[10] and pjh101, a pBR322 derivative which carries a chloramphenicol acetyl transferase gene expressed in *B. subtilis*[19].

The L- and D- isomers of the amino acids used were from Sigma (St. Louis MO). The restriction enzymes were purchased from New England Biolabs or Bethesda Research Laboratories; DNA polymerase I Klenow (large) fragment was from Boehringer, and T4 DNA ligase was from New England Biolabs. All the enzymes were used according to the suppliers' conditions.

Plasmid DNA was extracted from *E. coli* by the method of Birnboim and Doly[20]. High molecular weight chromosomal DNA from *B. subtilis* was prepared as described by Marmur[21]. DNA restriction fragments were separated on agarose or acrylamide gels as previously described[22]. For hybridization analysis DNA was digested with appropriate restriction enzymes, run on 1 percent agarose gel, depurinated by the method described by Wahl *et al.*[23], and transferred to nitrocellulose paper as described by Southern[24]. DNA probes were labelled with [gamma-P]CTP by nick translation[25]. Hybridization analysis DNA was digested with appropriate restriction enzymes, run on 1 percent agarose gel, depurinated by the method described by Wahl *et al.*[23], and transferred to nitrocellulose paper as described by Southern[24]. DNA probes were labelled with [gamma^{32}-P]CTP by nick translation[25]. Hybridization conditions with nick-translated DNA have been already described[26]. Restriction fragments to be sequenced were ligated into appropriate sites of M13 phage vectors mp8 or mp9[27], and DNA sequencing was carried out by the dideoxy method[28].

Example 1

Isolation of the dal structural gene

The initial strategy to obtain the *dal* structural gene was to transform a random bank of the *B. subtilis* genome, constructed in a plasmid vector which can replicate in *B. subtilis*, into a strain that carried a *dal* mutation and select for a Dal+ phenotype. Several attempts were not fruitful and a slightly different approach was attempted. Plasmid pJH101 (EP130, 756A) is a plasmid carrying a gene for chloramphenicol resistance which can integrate into the *B. subtilis* chromosome by homologous recombination; such integration events can be detectd by selecting for Cm^r because pJH101 cannot replicate extrachromosomally. Any non-replicative Bacillus plasmid is suitable for use in place of pJH101. If random fragments of the *B. subtilis* chromosome are ligated into pJH101, any fragment carrying the dal structural gene should integrate into the chromosome at the site of the chromosomal *dal* gene, and, presuming that the mutation is recessive, the transformant will be Dal+ and Cm^r. The region surrounding the integrated plasmid is then rescued in *E. coli*[7-8].

High molecular weight chromosomal DNA from *B.subtilis* 1168 (EP130, 756A) was partially digested with Sau3A, and ligated to BamHI digested pJH101. The ligation mixture was used to transform *B. subtilis* strain BG119 (available from the Bacillus Growth Stock Center as accession no. 1A4,[EF] or any other *dal*$^-$ *Bacillus* is suitable) with selection for both Cm^r and Dal+. Forty-two colonies were obtained which had acquired both the Cm^r and Dal + phenotype. In order to eliminate those Cm^r/Dal+ that were due to double transformation events, the 42 original transformants were combined into 6 pools, DNA was extracted, and these pooled DNAs

were used to transform BG119 at a low DNA concentration, 1ng/ml, which should give a very low frequency of double transformants. Two pools showed a cotransformation frequency to Dal+ and Cm^r greater than 50 percent, indicating that at least several of the isolates in these pools had an integrated plasmid very near to the *dal* structural gene. Six transformants from these two pools were selected and the linkage of the Dal+ and Cm^r phenotypes confirmed by PBS-1 transduction. Chromosomal DNA from the four isolates with the highest cotransduction frequencies was isolated and digested with HindIII. The digested DNA was diluted to a concentration of about 100 ng/ml to favor recircularization, ligated, concentrated by ethanol precipitation and used to transform *E. coli* strain MM294 (ATCC No. 31, 446). Analysis of plasmid DNA isolated from the transformants showed that the "rescued" plasmid from each isolate was different, indicating that each of the four isolates arose from an independent integration event. One of these four isolates, plasmid pDAL1, was able to transform strain BG119 to Dal+, indicating that it contained at least a portion of the *dal* structural gene, and this isolate was chosen for further study.

A restriction map of the DNA insert of pDAL1 is presented in Figure 1. The 1.75 kb EcoRI-EcoRI fragment, the 1.3 kb EcoRI-PvuII fragment and the 1.3 kb BglII-EcoRI fragment were individually subcloned into the replicating plasmid pBS42 (EP 120, 756A); these subclones were designated pDALsub1, pDALsub9 and pDALsub6, respectively (Figure 1).

Plasmid pDALsub1 was constructed by ligating the indicated EcoRI fragment of pDAL1 into the EcoRI site of pBS42, pDALsub9 was constructed by ligating the indicated EcoRI-PvuII fragment into the EcoRI and filled BamHI sites of pBS42 and pDALsub6 was constructed by ligating the indicated BGIII-EcoRI fragment into the EcoRI and BamHI sites of pBS42. It should be noted by way of explanation that the BglII and BamHI produce mutually cohesive terminii and that PvuII produced a blunt end which was ligated to BamHI sites that had been filled in by the Klenow fragment of DNA polymerase I. Plasmid pDALdeltaRI was constructed by a three way ligation of the indicated HindIII-EcoRI and EcoRI-PvuII fragments into the HindIII and filled BamHI sites of pJH101.

Plasmids pDALsub1 and pDALsub6 were able to transform strain BG119 to Cm^r and Dal+, indicating that the entire *dal* structural gene and sufficient regulatory signals for its expression were contained within the 1.3 kb BglII-EcoRI DNA fragment. Each of the purified fragments used to construct the subclones described above were also used to transform strain BG119, and each were able to transform BG119 to Dal+. As a matter of curiosity, this indicated that the *dal*-1 mutation of BG119 was localized in the 800 bp BglII-PvuII region common to all three fragments.

The DNA sequence of the 1.75kb EcoRI fragment was determined (Figure 2) and an open reading frame of 1169 nucleotides was determined which was contained within the 1.3 kb BglII-EcoRI fragment. The open reading frame encodes a potential protein of 43, 000 MR, which was concluded to be the *dal* structural gene, based upon the complementation data and transformation results.

Example 2

Isolation of a deletion mutant

It was preferable to delete the chromosomal *dal* structural gene from host cells as this would prevent any possible reversion of a chromosomal *dal*$^-$ mutation, and would eliminate any potential homologous recombination of a plasmid carrying the *dal* gene into the chromosome. The deletion mutation of the D-alanine racemase gene was done by a previously described method briefly diagrammed in Figure 3[9]. A plasmid containing the regions flanking the *dal* structural gene was constructed in pJH101 by ligating the 600bp of the HindIII-EcoRI DNA fragment located at the left end of pDAL1 to the 900 bp EcoRI-PvuII fragment located in the middle of pDAL1 (see Figure 1). This plasmid, pDALdeltaR1, upon integration through a double crossover event in the region of the D-alanine racemase gene would delete the entire *dal* gene. *B. subtilis* 1168 competent cells were transformed with pDALdeltaR1 selecting for Cm^r on TBAB plates containing 100 ug/ml of D-alanine. 5 *dal*$^-$ mutants were detected among 100 cm^r transformants tested for inability to grow on TBAB medium in the absence of D-alanine. One of them, BG2189, was isolated as a single colony and grown in the absence of chloramphenicol for 48 hours in PAB with 200 g/ml of D-alanine and 0.5 percent glucose. Appropriate dilutions of the culture were then plated on TBAB containing D-alanine and about 500 colonies were tested for loss of the CM^r. Two of the tested colonies were cm^s, and one of them, BG2190, after purification to a single colony, was examined by DNA blot hybridization to ensure that it carried the expected deletion. Figure 4 shows that when DNA from a strain carrying the wild type allele of the *dal* gene (lanes c and c') is compared to that from strain BG2190 (lanes e and e'), a smaller band hybridizes to the probe from strain BG2190, indicating a deletion in this area of the chromosome. The sizes of the bands correlate with those expected for a deletion of the 1.75 kb EcoRI fragment. This deletion mutation of the *dal* structural gene was designated delta *dal*-2. Complementation studies using the subcloned fragments of pDAL1 in pBS42 were repeated in strain BG2190 with identical results as those seen with strain BG119. Strains carrying the delta *dal*-2 mutation were unable to grow on complex media lacking D-alanine. However, they were able to grow on minimal media lacking D-alanine, but only in the absence of L-alanine. Strain BG119 showed this same capability, as had been previously reported for the *dal*-mutation[5].

Example 3

Plasmid stability

Plasmid stability studies were carried out with BG2198, a transformant strain BG2190 carrying the plasmid

pDALsub1 (which encodes the intact D-alanine racemase gene). Two independent experiments were performed. In one experiment a fresh isolate of the strain BG2198 on TBAB plates containing Cm and D-alanine, was inoculated into three 250 ml flasks, each containing 25 ml of PAB plus 0.5 percent of glucose. Culture A had no supplements, maintaining selective pressure for the Dal+ phenotype, culture B contained 200 ug/ml of D-alanine, giving no selective pressure, while culture C contained 200 ug/ml of D-alanine and 5 ug/ml of Cm, maintaining selective pressure for a Cm^r phenotype. At 24 and 48 hrs of growth at 37°C appropriate dilutions of the three cultures were plated on TBAB agar plates containing 100 ug/ml of D-alanine. After overnight incubation at 37°C individual colonies were tested for Cm^r and growth in the absence of D-alanine.

In the second experiment, which served to confirm the results obtained in experiment one, a plate culture of BG2198 on TBAB plus Cm was used to inoculate a 5ml culture in PAB which was incubated at 37°C to mid log phase. 100 ul of this culture were used to inoculate three different cultures A, B and C as described above, except that in this case PAB was replaced with NB. After 40 hrs at 37°C the cultures were plated and individual colonies tested for Cm^r and growth in the absence of D-alanine.

The results of both series of experiments, shown in Table 1 below, demonstrate that maintaining selective pressure for a Dal+ phenotype resulted in maintenance of the plasmid in the culture at levels comparable to or exceeding that seen when selection was for Cm^r. The absence of any selective pressure resulted in high frequency loss of the plasmid. None of the colonies tested showed segregation of the Cm^r and Dal+ phenotypes, indicating no structural instability of the plasmid at this level of analysis.

Table 2

Plasmid stability

	Experiment 1		Experiment 2
	24 hrs	48 hrs	40 hrs
Culture			
A	100% (600/600)	>99% (588/600)	>99% (288/300)
B	93% (372/400)	29% (44/162)	1% (2/158)
C	100% (420/420)	96% (155/162)	95% (142/150)

The phenotype of a strain carrying the delta *dal*-2 deletion suggest that there might be an alternative pathway to D-alanine that can be utilized under certain conditions. It had been previously noted that strains carrying the *dal*-1 mutation could grow on minimal media in the absence of D-alanine; however this growth was abolished in the presence of L-alanine or certain other L-amino acids[5]. It was not clear whether the *dal*-1 mutation was leaky and that slowly growing cells could synthesize enough D-alanine for their cell walls, or whether there was another pathway to provide D-alanine. Since the delta *dal*-2 mutation shows the same phenotype as the *dal*-1 allele, there must be an alternative pathway. In both *S. typhimurium* and *E. coli*, there are two alanine racemase genes[13,14]. One of the two genes is apparently constitutive at low levels and provides the D-alanine for cell wall biosynthesis, while the other is induced by L-alanine and is responsible for synthesis of D-alanine and cell growth. In rich media or in minimal media in the presence of L-alanine, the catabolic racemase would be repressed, the (presumably) constitutive enzyme is deleted, and the cells cannot grow due to lack of D-alanine.

The initial strategy to clone the alanine racemase gene was to construct a random bank of *B. subtilis* DNA in *E. coli*, using a plasmid vector that can replicate in both *E. coli* and *B. subtilis*. As mentioned above, this approach was unsuccessful. The probable reason was that *E. coli* strain MM294 carrying plasmid pDAL1 grows very slowly, and takes four days to form colonies when initially transformed. The cause of the slow growth is not known; however, its consequence is that in the random banks of *B. subtilis* DNA, plasmid pDAL1 would be either not present, or severely under-represented. However, pDalsub1, lacking a portion of the pDAL1 *B. subtilis* genomic DNA as described, is not growth inhibitory for MM294.

Example 4

Stable maintenance of a plasmid bearing DNA encoding a product protein

Plasmid pSPIF-IV[10], containing the human leukocyte interferon A gene under the control of the spac-I promoter, is digested with EcoRI in order to open the plasmid at its single EcoRI site. pDALsub1 is digested with EcoRI and the 1.75 kb racemase-containing EcoRI fragment recovered. The 1.75 kb fragment is ligated to the opened pSPIF-IV and transformed into *E. coli* MM294, which is then selected for Cm^r. Plasmid pDALLEIF was recovered from a resistant colony. After characterizing pDALLEIF by restriction enzyme analysis as having the gene in the proper orientation, *B. subtilis* 1168 is transformed with pDALLEIF, cultured and assayed as described previously[10], except that the culture medium will contain no Cm. pDALLEIF is maintained stably in *B. subtilis* 1168 even in the absence of Cm in the culture medium. pDALLEIF also is stably maintained in *E. coli* having inactivating mutants of both *E. coli* D-alanine racemase isozymes.

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CLAIMS

1. A method comprising providing a host cell which is unable to synthesize a cell wall constituent; providing a vector encoding (a) a protein complementing the genotypic deficiency of the host cell, thereby enabling the host cell to synthesize the cell wall constituent, and (b) a desired protein; culturing the host cell; and recovering the desired protein from the culture.

2. The method of claim 1 wherein the cell wall constituent is D-alanine.

3. The method of claim 1 or claim 2 wherein the protein for synthesizing the cell wall constituent is D-alanine racemase, L-glutamate racemase or D-alanyl-D-alanine ligase.

4. The method of claim 3 wherein the protein is D-alanine racemase.

5. The method of any one of claims 1 to 3 wherein D-alanine racemase, L-glutamate racemase or D-alanyl-D-alanine ligase are deleted from the host cell genome or are expressed in the host cell in enzymatically inactive form.

6. The method of any one of the preceding claims wherein the host cell is a bacterium.

7. The method of claim 6 wherein the bacterium is a Bacillus species.

8. A method of any one of the preceding claims wherein the desired protein is a mammalian protein.

9. A method comprising providing a host cell which is unable to grow or survive in a hypotonic complex medium free of substances which are toxic to the host cell; providing a vector encoding (a) a complementary protein that enables the cell to grow and survive in such medium and (b) a product protein; transforming the host cell with the vector; culturing the host cell on the medium; and recovering the desired protein from the culture.

10. The method of claim 9 wherein the host cell is a bacterium.

11. The method of claim 10 wherein the complementary protein is an enzyme required in the synthesis of peptidoglycan.

12. The method of claim 11 wherein the enzyme is D-alanine racemase, D-glutamate racemase or D-alanyl-D-alanine ligase.

13. The method of any one of claims 9 to 12 wherein the medium does not contain a predetermined deficiency of an L-amino acid.

14. The method of any one of claims 9 to 13 wherein the toxin is an antibiotic or metal ion.

15. The method of any one of claims 9 to 14 wherein the nitrogen source in the medium is undefined and of non-bacterial origin.

16. A method comprising providing a bacterial cell which enzymatically synthesizes a cell wall, and mutating the genome of the cell in order to create a predetermined deletion in the cell DNA responsible for the expression of an enzyme necessary for the synthesis of the cell wall whereby the cell is rendered phenotypically unable to synthesize peptidoglycan.

17. The method of claim 16 wherein the mutated cell is cultured in an isotonic fermentation medium.

18. A method according to any preceding claim substantially as any described nd exemplified herein.

19. The cell of any one of the preceding claims wherein the enzyme is D-alanine racemase.

20. The cell produce by the method of any one of claims 1 to 18.

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